CHARACTERIZING THE ROLE OF A PHOSPHORELAY IN THE CIRCADIAN REGULATION OF THE OS MAPK PATHWAY IN *NEUROSPORA CRASSA*

A Thesis

by

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ABSTRACT

Circadian regulation of Mitogen Activated Protein Kinase (MAPK) pathways provides a priming mechanism to allow organisms to anticipate daily environmental stresses such as heat, light, desiccation and osmotic stress. In *Neurospora crassa*, the OS MAPK pathway, which is homologous to the p38 MAPK pathway in mammals and the Hog pathway in yeast, is important for adaptation to osmotic stress. The OS pathway consists of two modules: a sensory phosphorelay and a downstream MAPK module. Previous work in our lab demonstrated that under constant environmental conditions, the circadian clock controls daily activation of the MAPK OS-2, through direct transcriptional regulation of the MAPKKK, OS-4. Although this signaling is necessary for rhythmic activation of OS-2, it is not known if it is sufficient for robustness in the amplitude of the rhythms. An upstream phosphorelay, consisting of a sensor histidine kinase (OS-1), a phosphotransferase (HPT-1) and a response regulator (RRG-1), is required for acute stress signaling to the MAPK cascade to activate OS-2. We discovered that the levels of RRG-1 are clock-controlled, suggesting that the phosphorelay functions to regulate rhythms in activation of OS MAPK cascade. To test this idea, I generated constructs with mutations in the phosphorylation site of RRG-1 that will abolish rhythmic activation of the phosphorelay components. These strains will be used to determine if loss of temporal activation of the phosphorelay alters rhythmic accumulation of active OS-2. In addition, transcriptional reporter fusion construct was generated to test if *rrg-1* transcription is under the control of the circadian clock. To

detect phosphorylation of RRG-1, phos-tag coupled with SDS-PAGE gels was used. This procedure will be utilized in future studies to test if phosphorylation of RRG-1 is rhythmic. Further, two upstream histidine kinases (NCU07221 and NCU00939) were validated as direct targets of the white collar complex (WCC). Tagged versions of these proteins were generated, which will be used in future studies to determine if these histidine kinases accumulate with a circadian rhythm to further understand their role in the clock regulation of the OS MAPK phosphorelay.

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NOMENCLATURE

ccg	clock controlled gene
DD	constant dark
DNA	deoxyribonucleic acid
FFC	FRQ-FRH complex
FRH	FRQ-interacting RNA-helicase
FRP	free running period
frq	frequency
HK	histidine kinase
HPT	histidyl phosphotransferase
IP	Immunoprecipitation
kb	kilobases
LL	constant light
LRE	light response element
LUC	luciferase
MAPK	mitogen activated protein kinase
mRNA	messenger ribonucleic acid
ORF	open reading frame
OS	osmo sensing
PAGE	polyacrylamide gel electrophoresis
PAS	PER-ARNT-SIM

PCR	polymerase chain reaction
RRG	response regulator
SDS	sodium dodecyl sulphate
wc	white collar
WCC	white collar complex
WT	wild type

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1. INTRODUCTION

1.1 Overview of circadian clocks

Circadian clocks are endogenous time-keeping machineries that help organisms to synchronize their daily biological activities to the 24 h light-dark cycles imposed by the rotation of earth. Organisms ranging from cyanobacteria to mammals exhibit circadian rhythms in physiology, behavior, metabolism and gene expression. Circadian clocks enable the organisms to co-ordinate their biological functions to the most appropriate time of the day. Temporal co-ordination is important in terms of energy expenditure in biological systems. Most biological processes are energetically expensive and hence shutting them down at the time of day when their operation is not necessary is advantageous (1, 2). In addition circadian clocks allow organisms to anticipate and respond to predictable changes in the environment. For example, circadian clocks in plants control the expression of genes involved in photosynthesis such that they peak in the morning and their expression is lowest in the night (3). Circadian clocks therefore confer an adaptive advantage to organisms.

1.2 Properties of circadian rhythms

Circadian rhythms are characterized by three important properties as follows (4):

a) Circadian rhythms are self-sustaining oscillations that persist with an endogenous free running period (FRP) of approximately 24 h in the absence of an environmental cue.

b) Circadian rhythms can be entrained to external environmental cues (zeitgebers), such as light and temperature. As the FRP is close to 24 h, entrainment to zeitgebers allows the oscillator to reset to the external time.

c) Circadian rhythms are temperature compensated. This means that the FRP of the circadian rhythm stays relatively constant across a physiologically relevant range of temperatures.

1.3 Molecular basis of circadian rhythms

In most eukaryotes, the circadian oscillator functions by means of a core molecular feedback loop which drives rhythmic expression of genes. The core molecular loop consists of both positive and negative elements; the interaction of these components generates oscillations with an approximately 24 h period. The positive elements and the negative elements perform opposing functions: the positive elements stimulate the expression of negative elements, which in turn inhibit the activity of the positive elements. The interplay between positive and negative elements gives rise to a self-corrective negative feedback loop in which the negative element inhibits its own expression. Circadian clocks are composed of the following three parts: (i) input pathways, which consist of sensory systems that signal environmental cues to entrain the oscillator, (ii) one or more oscillators that keep time, and (iii) output pathways that signal time of day information from the oscillators to control overt rhythmicity (5).

1.4 *Neurospora crassa* as a model system to study circadian clocks

Neurospora crassa is a well-characterized model system to study circadian rhythms. This is due to several reasons, including availability of sophisticated molecular and genetic techniques, a sequenced and annotated genome (6), a gene knockout collection (7, 8), and an easily assayable circadian rhythm in asexual spore production, called condiation (9). Conidiation rhythms are assayed by culturing *N. crassa* in long glass assay tubes called race tubes (10, 11), and in strains containing *ras-1*^{bd} mutation (9). This mutation helps to clarify the conidial banding pattern in closed culture tubes, and provided the opportunity to identify mutations that altered the conidiation rhythm (12-14). Such genetic approaches led to the identification of core clock genes (15). Molecular rhythms can be assayed by growing *N. crassa* in liquid cultures in constant dark (DD) and harvesting the cells at 2-4h intervals over the course of 2 days. The cells are harvested, ground in liquid nitrogen, and RNA and proteins are extracted from ground tissue (16). More recently, advances in technology led to development of codon modified luciferase reporter systems. The use of transcriptional and translational luciferase reporter fusions allows for easy monitoring of rhythms in mRNA and protein, respectively by using bioluminescence counters that continuously measure luciferase activity in constant conditions (17-19).

1.5 The *Neurospora* circadian clock

In eukaryotes, the primary mechanism of the circadian clock resides with the core molecular oscillator that forms a negative feedback loop and consists of both postive and

negative elements (Figure 1). In *N. crassa,* the negative arm of the feedback loop consists of two proteins, FREQUENCY (FRQ) and *frq*-INTERACTING RNA HELICASE (FRH), which interact to form a complex known as the FFC (20-22). The positive arm consists of a heterodimeric complex (WCC), which is formed by the interaction of two proteins WHITE COLLAR-1 (WC-1) and WHITE COLLAR-2 (WC-2) (23, 24). In the late subjective night, the WCC binds to the Clock box (C box) in the *frq* promoter, and activates *frq* transcription (25). In the early subjective morning, *frq* mRNA levels peak and FRQ proteins levels increase. FRQ protein dimerizes and forms a complex with FRH (26, 27). The FFC inhibits the activity of the WCC in the nucleus, which ultimately causes inhibition of *frq* transcription (20-22). As FRQ protein accumulates, it is progressively phosphorylated and degraded (28, 29). When FRQ is fully degraded, WCC is no longer inhibited by FFC, and reactivates *frq* transcription to initiate a new circadian cycle. FRQ also positively regulates the levels of WC-1 proteins forming an interconnected positive loop (30-32).

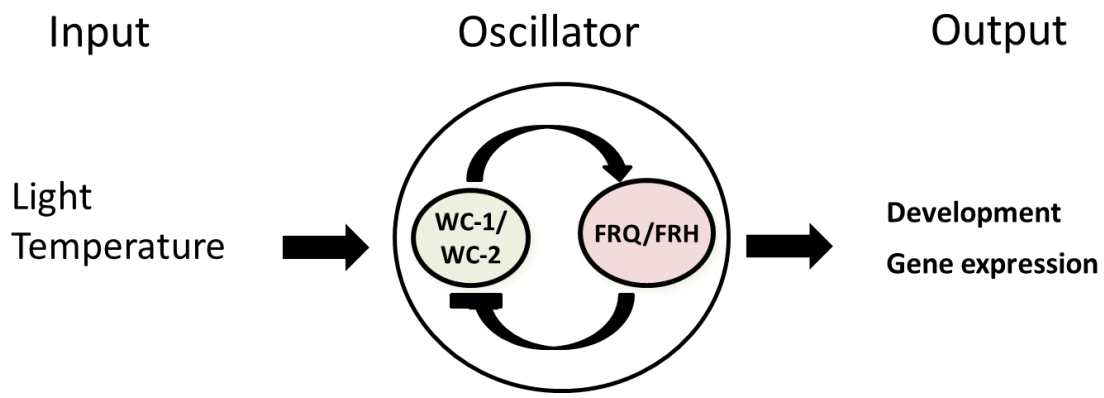


Figure 1 The circadian clock organization in *N. crassa.*

1.6 Circadian output pathways in *Neurospora*

Clock controlled genes (*ccgs*) were first identified in *N. crassa* using substractive hybridization of morning versus evening specific mRNAs which led to the identification of the genes *ccg-1* and *ccg-2* (33). In a subsequent study, additional *ccgs* were identified by using differential screens of time-of-day specific cDNA libraries (34). Expressed sequence tags were used to identify additional *ccgs* that peaked in the subjective morning as was the case in previously identified *ccgs* (35). Recently genome wide studies have been carried out to characterize the circadian output pathways by identifying genes involved in these pathways. The first of these studies was done using

microarrays which provided useful insights into the extent of clock control of gene expression. Microarrays were generated using EST libraries from 3 major stages of *N. crassa* lifecycle including the vegetative, conidial and sexual phases. This study identified approximately 145 *ccgs* that peaked at all times of the day but primarily in the late night to early morning phase. Functional characterization of these genes revealed that clock regulation is wide spread and includes pathways involved in cell signaling, stress responses and metabolism (36). In addition, ChIP-seq of the WCC was carried out to identify direct targets of the WCC. TFs including both activators and repressors were enriched among the WCC targets. This information could perhaps explain the mechanism of clock regulation at various phases over the circadian cycle (37). Recently, RNA-seq was carried out with 2 h temporal resolution and it was found that approximately 40% of the transcriptome is expressed under the control of the circadian clock. Functional analysis of the clock-controlled genes revealed an enrichment for genes involved in pathways such as metabolism, protein synthesis and stress responses (38). Together, these data demonstrated that the circadian clock in *N. crassa* regulates a wide array of biological processes including metabolism, development, cell signaling and stress responses (39, 40).

1.7 MAPK signaling pathways and circadian clocks

In addition to the day-night cycle imposed by Earth's rotation, organisms also encounter unpredictable changes in the environmental conditions. These include changes in

osmotic conditions, nutrient starvation, heat shock and desiccation. Organisms use conserved signaling pathways to sense and respond to such stimuli, including employing highly conserved MAPK signaling pathways (41). The MAPK pathways are cascades of hierarchical proteins: at the first tier of the cascade is the MAP kinase kinase kinase (MAPKKK) followed by the MAP kinase kinase (MAPKK) and the MAP kinase (MAPK). The MAPKKK is activated by autophosphorylation in response to an activating signal. Phosphorylated MAPKKK phosphorylates and activates the MAPKK, which ultimately activates the MAPK (42). Upon activation, the MAPK activates a number of effector molecules, including TFs, translation factors, kinases, and chromatin remodeling factors, which are required for the expression of various stress responsive genes (43, 44). In plants and fungi, two-component systems consisting of sensor histidine kinases and response regulators constitute the main sensory apparatus, which in turn activates the MAPK cascades (41, 45-47), whereas higher eukaryotes employ more complex mechanisms, involving a large number of sensory receptors that activate multiple MAPK cascades (48).

Recently it has been demonstrated that MAPK pathways regulate a wide variety of processes that are also regulated by the circadian clock. Some examples of processes that are regulated by MAPK pathways and the clock include growth, cell division, differentiation, morphogenesis, mating, and apoptosis (41, 42, 49). In addition, several studies have suggested a link between the circadian clock and MAPK signaling pathways (43, 50-54).

In *N. crassa*, the circadian clock regulates the activity of all three MAPKs: OS-2, MAK-1 and MAK-2 (50, 55). MAK-1 is homologous to Slt2 in *Saccharomyces cerevisiae* and the extracellular signal-regulated kinase (ERK 1/2) in mammals, and in *N. crassa* is required for maintenance of cell wall integrity. MAK-2, which is a homolog of *S. cerevisiae* Fus3 and mammalian ERK 1/2, is important for initiation of the sexual cycle, vegetative growth and hyphal fusion (56-59). Similar to its counterpart in yeast (Hog-1p), *N. crassa* OS-2 MAPK is necessary for responses to osmotic stress (60-65).

Circadian regulation of stress response pathways in fungi is thought to provide a mechanism to prime the stress response to prepare for daily exposure to acute environmental stressors, such as heat and desiccation (43). In higher eukaryotes, circadian regulation of MAPK pathways could be involved in temporal regulation of biological processes; for instance, coordinating feeding behavior with processes involved in energy metabolism. In addition, there is increasing evidence which indicates that many of the same types of cancers are associated with disruption of both circadian clocks and MAPK pathways (66-69). For instance, there has been increasing evidence that suggests that the circadian clock regulates processes such as cell cycle and DNA damage responses (70, 71). WEE1, a cell cycle kinase was identified as one of the coupling components connecting the cell cycle and the circadian clock in the mouse liver (72) and in *N. crassa* (73). Thus, understanding how the circadian clock controls the activity of MAPK pathways may shed new light on treatments for these cancers.

1.8 The OS pathway in *Neurospora crassa*

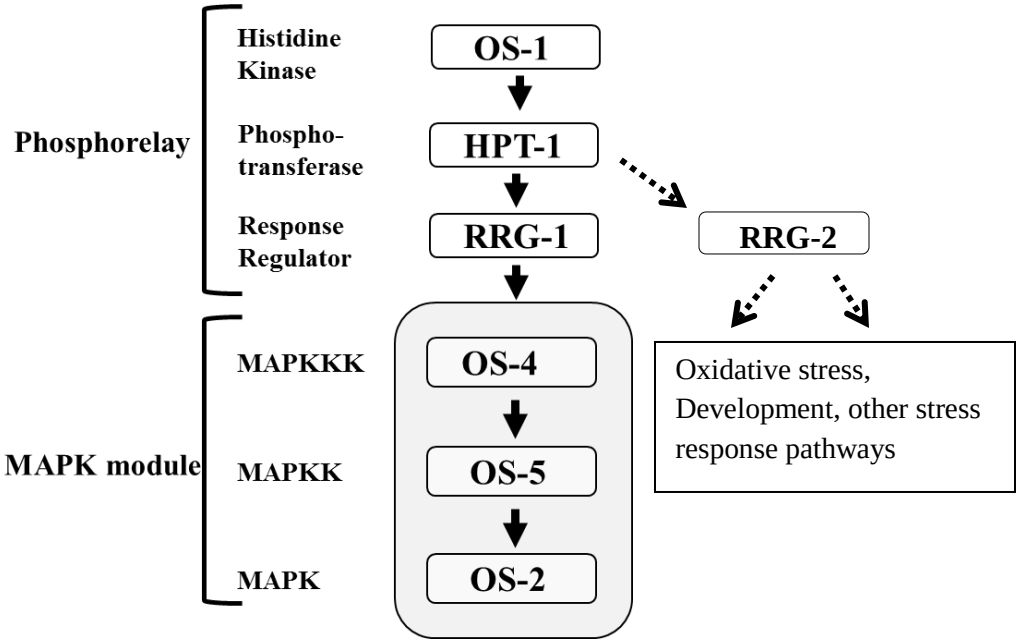


Figure 2 The OS pathway. The two modules of the OS pathway are shown.

The OS pathway is important for mediating the response to osmotic stress in *N. crassa*, similar to the Hog-1 pathway in *S. cerevisiae* (60, 64). The OS pathway consists of two modules: a sensory phosphorelay and a downstream signaling cascade (Figure 2). The two component phosphorelay serves as a sensory apparatus to detect environmental signals and transduce the signal downstream to activate the MAPK cascade. In *N.*

crassa, the phosphorelay consists of a sensor histidine kinase (OS-1), which is activated upon osmotic stress (74). Activated OS-1 signals to a histidyl phosphotransferase (HPT-1), and subsequently to the response regulator (RRG-1) (63). RRG-1 then activates the MAPKKK OS-4 by an unknown mechanism. Although its counterpart, the *sln1-ypd1-ssk1* phosphorelay is comparatively well characterized, the mechanistic operation of the OS pathway in *Neurospora* is not well understood. In yeast, the absence of osmotic stress results in continuous phosphotransfer between the components of the phosphorelay. Upon receiving an osmotic stress signal, the histidine kinase stops auto-phosphorylating, and the unphosphorylated form of the response regulator activates the MAPKKK by binding to the N-terminal regulatory domain of the MAPKKK (45, 63, 75, 76).

The *N. crassa* OS pathway was identified as an output pathway from the clock using a genetic selection for mutations that disrupted rhythms in *ccg-1* and *ccg-2* expression (39). Previous work in our lab demonstrated that phosphorylation of OS-2 is clock-controlled, and the levels of phosphorylated OS-2 peak during the subjective morning (50). It was also found that circadian regulation of OS-2 activity occurs via direct transcriptional control of the *os-4* promoter by the WCC (37, 43). Deletion of the WCC binding site (known as the light response element (LRE) abolished *os-4* mRNA and OS-4 protein rhythms, as well as rhythmic phosphorylation of OS-2 (43). It was also shown that the circadian clock regulates HPT-1, a key component of the phosphorelay (43). However, it was not determined if circadian regulation of HPT-1 is important for

rhythmic activation of the MAPK cascade, or if HPT-1 functions to activate other stress response pathways. Additionally, it was shown that the OS pathway-controlled transcription factor (TF), ASL-1, regulates rhythmic transcription of genes involved in osmotic stress responses, including genes involved in glycerol production (77). Finally, recent studies demonstrated that activation of a conserved eukaryotic translation elongation factor (eEF-2) is clock-controlled and that rhythmic phosphorylation of eEF-2 depends on the rhythmic activation of the MAPK, OS-2 *(Caster et. al., in review)*. Together, these data suggested that the OS MAPK pathway in *N. crassa* is involved in the regulation of processes involved in stress response, transcription and translation.

Although the molecular mechanisms of MAPK pathway activation are extensively studied in organisms ranging from yeast to mammals, the role of the clock in circadian regulation of these pathways is not understood. In particular, a role for the upstream phosphorelay in rhythmic control of OS-2 activity has not been studied, in part due to the lack of available tools to probe the phosphorelay. My goal was to develop the tools needed to understand the mechanistic details of OS MAPK pathway activation in *N. crassa*. Understanding signal transduction through the phosphorelay will give important insights into the operations of cellular signal transduction pathways in fungi. Because components of the phosphorelay are not conserved in higher eukaryotes, understanding how the phosphorelay system works might also provide insights into how to target the pathway with antifungal drugs.

2. MATERIALS AND METHODS

2.1 Strains and culture conditions

N. crassa wild-type (WT) strain 74OR23-1 (FGSC 2489) was obtained from the Fungal Genetics Stock Center. For ChIP, already available strains *bd*$^{-}$ (DBP 369) and *bd*$^{-}$*;Δwc*-1 (DBP 257) were used. WT and *bd*$^{-}$ strains were grown and maintained on Vogel's minimal medium (1X Vogel's salts, 2% glucose). Strains containing hygromycin resistant gene cassette (*hph*) were maintained on Vogel's minimal medium containing 200 μg/mL hygromycin B (Calbiochem, Darmstadt, Germany). Mycelial mats were made by inoculating a loopful of conidia from slants to 20mL of 1X Vogel's 2%glucose 0.5% arginine in petri dishes. For phos-tag gel analysis, immunoprecipitation and sucrose gradient analysis, discs of mycelial mats cut with core-borer (size 3) were inoculated into 125mL Erlenmeyer flask containing 75mL 1X Vogel's 2% glucose 0.5% arginine medium. The cultures were grown in constant light (LL) at 30˚ C for 40 h and then harvested in liquid nitrogen. For light induction, cultures were grown in LL 30˚ C for 24 h and then transferred to constant darkness (DD) at 25˚ C for 24 h following which cultures were exposed to light (25˚ C) for 0, 15, 30 and 60 minutes and then harvested.

2.2 Strain and plasmid construction

To determine the rhythmicity of RRG-1, a reporter strain expressing a RRG-1::LUC translational fusion construct was generated. The RRG-1::LUC construct was made

using 3-way PCR strategy with a fully codon-optimized luciferase gene (17, 18), and was then transformed into wild type *N. crassa* strain 74OR23-1 to replace the endogenous *rrg-1* gene with the RRG-1::LUC fusion construct by homologous recombination. Promoter-driven luciferase reporter construct for *rrg-1* was generated using recombinational cloning in yeast as previously described (78). Five PCR fragments (5' of *csr*, promoter region of the gene, codon optimized *luc* sequence (17, 18), 3' UTR of gene, and 3' of *csr*) were co-transformed with gapped plasmid (pRS426) digested with *Xho*I and *Bam*HI into yeast strain FY2 (MATα, ura3-52). The yeast transformants were screened by PCR and the full length product was amplified from yeast DNA using the universal 5' and 3' *csr* primers. For generating the phosphorylation site specific mutants of RRG-1, PCR based site directed mutagenesis strategy was used. Two PCR products were amplified in separate PCR reactions each using a universal primer and a mutagenic primer. In the subsequent PCR reaction, the two products from the first round were used as templates for PCR, and the full-length fragment containing the desired substitution was amplified using the universal primers. To generate the HA tagged constructs of NCU07221 and NCU00939, 3-way PCR was performed. The constructs were then transformed into WT *N. crassa* strain (FGSC 2489) whereby the endogenous genes were replaced by the fusion constructs by homologous recombination. The transformants were screened by Western blot analysis using anti-HA antibody and integration at the endogenous locus was validated by PCR.

Table 1 provides a list of all the primers that were used to make the above constructs.

Table 1 Primers used in the construction of the fragments generated in this study

Strain	Forward Primer/Reverse Primer	Purpose	Size of the amplicon (Kbps)
RRG-1::LUC	**5' CCTTGCGCTTTCACACAC 3'** **5'CTTGATGTTCTTGGCGTCCTCCTTATCGTCGCTCGAAGT 3'**	**To amplify ORF of *rrg-1***	**3.5**
	5'ACTTCGAGCGACGATAAGGAGGACG CCAAGAACATCAAG 3' **5'AGATAAGAGACCGCCAGGTGTCAGACGGCGATCTTGCCGCC 3'**	**To amplify the codon optimized LUC fragment**	**1.8**
	5'GGCGGCAAGATCGCCGTCTGACACC TGGCGGTCTCTTATCT 3' **5'CGTCCGCTCACTTAATAGGC 3'**	**To amplify the 3' UTR**	**0.6**
P_{rrg-1}::luc	**5'CGGAATTATACGATTTAGGTGACTGC AGGGCGGAGATGTCAACCTGGAG3'** **5'CTTGATGTTCTTGGCGTCCTCCATGT GGTTTTGAGGAGGGCTGT3'**	**To amplify the promoter region of *rrg-1***	**2.4**
	5'CAAGAAGGGCGGCAAGTCCAAGCTC TGACACCTGGCGGTCTCTTATCT3' **5'TAGGTATTCTATAGTGTCGGATCCTC TAGCGTCCGCTCACTTAATAGGC3'**	**To amplify the 3' region of *rrg-1***	**0.6**

Table 1 (Continued)

Strain	Forward Primer/Reverse Primer	Purpose	Size of the amplicon (Kbps)
RRG-1 D to E mutant	5'AGCCAGAGAAGAAGGGAACCCACAT 3' 5'GCAGCTGAATTTCCATCAACACCAAG TGAAAG 3'	To amplify fragment 1 of RRG-1 D to E mutant construct	5.0
	5'TTGGTGTTGATGGAAATTCAGCTGCC CATCATGA 3' 5'ACGGCAAGACCATCTTTGCCTTGG 3'	To amplify fragment 2 of RRG-1 D to E mutant construct	4.0
RRG-1 D to A mutant	5'AGCCAGAGAAGAAGGGAACCCACAT 3' 5'TTGGTGTTGATGGCCATTCAGCTGCC CATCATGA 3'	To amplify fragment 1 of RRG-1 D to A mutant construct	5.0
	5'GCAGCTGAATGGCCATCAACACCAA GTGAAAG 3' 5'ACGGCAAGACCATCTTTGCCTTGG 3'	To amplify fragment 1 of RRG-1 D to A mutant construct	4.0

2.3 Protein extraction and Western blot assays

Total protein extraction, protein quantification and Western blot assays were done as previously described (43). For sucrose gradient analysis, glycerol was not added to the extraction buffer. To detect HPT-1::FLAG, 12% SDS PAGE gels were used. The membranes were blocked with 5% non-fat milk in tris-buffered saline with 0.1% tween (TBST) overnight at 4˚ C and then incubated with rabbit anti-DYKDDDDK antibody (2368, Cell Signaling Technology, Beverly, MA) at a dilution of 1:1000 in 5% bovine serum albumin (BSA)-TBST. After an overnight incubation at 4˚ C, membranes were washed 6 times for 10 min each in TBST and then incubated with a 1:10,000 dilution of secondary antibody [(goat anti-rabbit IgG(H+L)–horseradish peroxidase (HRP) conjugate; Bio-Rad, Hercules, CA] in 5% BSA-TBST at 4˚ C overnight. Membranes were washed 6 times for 10 min each in TBST. Immunoreactivity was then visualized with a Super Signal West Pico chemiluminescence detection kit (Thermo Scientific, Waltham, MA). For RRG-1::HA, the same procedure as above was followed, except that an 8% SDS PAGE gel concentration was used membranes were incubated with primary rat anti-HA antibody (11867423001, Roche) at a dilution of 1:1000 in 5% non-fat milk in TBST and secondary anti rat IgG peroxidase (HRP) conjugate (Bio-Rad, Hercules, CA) was used at a dilution of 1:10,000 in 5% non-fat milk in TBST. Immunoreactivity was visualized with a Super Signal West Femro chemiluminescence detection kit (Thermo Scientific, Waltham, MA). For RRG-2::V5 detection, the primary antibody used was mouse anti-V5 (Invitrogen, Carlsbad, CA) at a dilution of 1:5000 with 5% non-fat milk in TBST.

2.4 Phos-tag SDS-PAGE analysis of RRG-1::HA

Phos-tag acrylamide was obtained from Wako chemicals (Richmond, VA). 8% SDS PAGE gels were made with the addition of 100 µL 5mM phos-tag acrylamide solution and 100 µL 10mM $MnCl_2$ solution. To verify the phosphate-specific signal, protein extracts were treated with λ-protein phosphatase (New England BioLabs, Ipswich, MA) as indicated. 100 µg total proteins were loaded on to the gel for each sample. The gels were run at a constant voltage of 40V for 6 h (until the dye front ran out of the gel). Before transfer the gels were washed in 20 mL Western transfer buffer with 100 µL ethylene diamine tetraacetic acid (EDTA) for 30 min at 4˚ C and Western blot analysis was carried out as mentioned previously.

2.5 Luciferase assay

Luciferase levels were measured from conidia inoculated into 96-well microtitre plates containing 1X Vogel's salts, 0.01% glucose, 0.03% arginine, 0.1 M quinic acid, 1.5 % agar and 25µM firefly luciferin (LUCNA-300; Gold Biotechnology, St. Louis, MO), pH 6.0, For synchronization of the cells, the plates were incubated in LL 30°C for 24 h, and then transferred to DD 25°C for 5 days. Bioluminescence was measured using EnVision multilabel plate reader (PerkinElmer Life Sciences, Boston, MA). Raw luciferase data were analyzed for period, phase, and amplitude determination using BioDARE (Biological Data Repository) (http://www.biodare.ed.ac.uk).

2.6 Immunoprecipitation of HPT-1::FLAG

For immunoprecipitation, EZview Red Anti-Flag Anti Affinity gel (Sigma-Aldrich, St. Louis, MO) was used. The gel beads were mixed until they were uniformly suspended. 30 µL of the slurry was dispensed into Eppendorf tubes with a wide orifice pipette tip. The beads were washed thrice with 500 µL TBS (50 mM Tris HCl, 150 mM NaCl, pH 7.4). Each wash consisted of adding TBS to the beads, vortexing briefly followed by centrifugation for 30 s at 8,200 x g. The supernatant was then discarded. The beads were kept on ice during the entire procedure. After the final wash, 8 mg total protein was added to the beads for each sample and the total volume was made up to 1000 µL. The tubes were then agitated gently on a nutator for 6 h at 4˚ C. After incubation, the suspension was centrifuged at 8,200 x g for 30 s. The supernatant was removed to a fresh tube for further analysis. The beads were washed with TBS thrice similar to the washing step pre-incubation. After the final wash, the proteins were eluted from the beads by adding 2X sample buffer for Western blot analysis directly to the beads and boiled for 5 min. The samples were briefly centrifuged to precipitate the beads and the supernatant was loaded on to the gel.

2.7 Sucrose gradient analysis

Sucrose gradient analysis was performed using the protocol as described previously (79). After ultracentrifugation, the proteins were precipitated using chloroform-methanol

precipitation method. The precipitants were solubilized in 2X SDS PAGE sample buffer for Western blot analysis.

2.8 WC-2 ChIP and analysis of ChIP

Chromatin Immunoprecipitation (ChIP) was performed as described previously (43). Immuno-precipitated DNA was analyzed by absolute quantitative PCR using Fast SYBR Green Mastermix and Fast 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, CA). For each of the primer sets, a standard to be used for absolute quantification was generated by amplifying a PCR product from genomic DNA that includes the genomic region to be analyzed (43). The standard PCR product was purified using the PCR Clean-up Kit (Qiagen, Valencia, CA) and quantitated using gel electrophoresis and ethidium bromide staining compared to a DNA sample of known concentration. The concentration of the standard was determined by densitometric comparison between the two samples. Two sets of primers were used for the analysis: one set flanking the WC-2 binding sequence in the promoter of each gene and the second set in the internal region within the gene. The primer sequences are as follows:

<u>WCC binding region for NCU00939</u>

Forward primer 5’ GAGTCGTGATGGAGTTTGGAGGA 3’ and reverse primer 5’ GACATCTCTGCCGATGTCTCG 3’

<u>Non-specific binding control for NCU00939</u>

Forward primer 5’ GTCATGGACGGAATGAGTTG 3’ and reverse primer 5’ CCTAGTAGTCGTGTACGCTA 3’

WCC binding region for NCU07221

Forward primer 5’ CTGGTTTCATCCGTCTCAGC 3’ and reverse primer 5’

TCGTGACTCGATTTTGTTGG 3’

Non-specific binding control for NCU07221

Forward primer 5’ TTTCTCAGTCAGCTCCGATG 3’ and reverse primer 5’

GGACGGACTTTGGAAGGTTA 3’

3. CONSTRUCTION OF TOOLS TO STUDY THE ROLE OF PHOSPHORELAY IN THE CIRCADIAN REGULATION OF THE OS MAPK PATHWAY

3.1 Introduction

The activity of the MAPK OS-2 has been shown to be rhythmic with a peak at subjective morning (50). This regulation occurs by direct transcriptional control of the gene encoding the MAPKKK OS-4 by the WCC (43). It was demonstrated that the WCC binds rhythmically to the promoter of *os-4*. This leads to rhythmic transcription of *os-4* mRNA, rhythmic accumulation of OS-4 protein, and subsequently leads to rhythms in OS-2 activity. In a mutant that lacks the binding site for the WCC at the *os-4* promoter (ΔLRE1-3), the accumulation of *os-4* mRNA, and phosphorylation of OS-2, was arrhythmic (43). Thus, the transcriptional control of *os-4* by the WCC is necessary for rhythms in OS-2 MAPK activation. In addition, previous work demonstrated that components of the phosphorelay upstream of the MAPK module are clock-controlled. HPT-1 is a central component of the phosphorelay, and both *hpt-1* mRNA and HPT-1 protein accumulate rhythmically (43). The peak of HPT-1 occurs during the subjective night, opposite to the day time peak in OS-2 phosphorylation. Assuming that the levels of phosphorylated HPT-1 and its response regulator target RRG-1 also peaks in the night; these data support the idea that the phosphorylated form of RRG-1 inhibits activation of the MAPK module, and a model whereby activity of the phosphorelay at

night inhibits activation of the downstream MAPK. Alternatively, during the day, the levels and phosphorylation of the phosphorelay components are predicted to be low, allowing daytime activation of the MAPK pathway. Together, we predict that the anti-phasic regulation of the two modules of the OS pathway helps to prime the stress response to the day, a time when the cells are more susceptible to stressful environment. In addition, in cells lacking RRG-1, OS-2 phosphorylation is undetectable, suggesting that RRG-1 is necessary for activation of the OS MAPK module (50). Taken together, these data suggest that the regulation of the OS MAPK pathway occurs at multiple levels. Based on this idea, I hypothesized that the multi-layer regulation of the OS pathway by the circadian clock contributes to robustness in the amplitude of the P-OS-2 rhythms.

3.2 Construction of phosphorylation site mutants of RRG-1

In the phosphorelay mechanism, signal transduction occurs by transfer of a phosphate group from the sensor HK OS-1, downstream to the response regulator RRG-1 via the intermediate HPT-1 (45). In order to eliminate the clock signal from the phosphorelay to the OS MAPK module, and to examine if the phosphorylated or unphosphorylated form of RRG-1 is responsible for activating the MAPK module, I proposed to construct mutant versions of RRG-1 in which RRG-1 is either constitutively phosphorylated or constitutively non-phosphorylated. From studies in yeast, it has been demonstrated that the non-phosphorylated version of the response regulator activates the MAPK cascade

upon osmotic stress (75). However, the mechanism by which *Neurospora* RRG-1 activates the OS-4 MAPKKK is mostly unknown.

Phosphorylation of RRG-1 occurs at a conserved aspartate (D-921) residue (63). In *S. cerevisiae*, mutation of the response regulator protein (Ssk-1p) containing either aspartate to alanine or aspartate to glutamate substitutions to prevent or mimic phosphorylation, respectively, were lethal due to the hyper-activation of the Hog-1 MAPK (75). Based on this observation it was concluded that these amino acid substitutions rendered the protein non-phosphorylatable and constitutively active. To test if the same mutations result in similar RRG-1 functions in *N. crassa*, I constructed phosphorylation site-specific mutations of RRG-1 in which the conserved aspartate (D-921) residue was replaced by either alanine or glutamate. This was accomplished using PCR-based site-directed mutagenesis as depicted in the schematic in Figure 3A. Two PCR products were amplified in separate PCR reactions each using a universal primer and a mutagenic primer (Figure 3B). In the subsequent PCR reaction, the two products from the first round were used as templates for PCR, and the full-length fragment containing the desired substitution was amplified using the universal primers. The product obtained from PCR 3 was loaded on 0.8% agarose gel. Figure 3C depicts the full-length product obtained from PCR 3 corresponding to the correct size of 9 Kb. This fragment was gel purified and cloned into PCR Blunt vector. The mutation was verified by sequencing the plasmid DNA containing the inserted fragments. This construct can now be transformed into *N. crassa* and the resulting transformants examined for levels

and rhythms in the accumulation of phosphorylated OS-2. From preliminary experiments transforming the constructs into WT N. crassa strain, no viable transformants were obtained, suggesting that *N. crassa* RRG-1 probably behaves similar to the yeast Ssk2p, such that the unphosphorylated version of RRG-1 activates the MAPK OS-2.

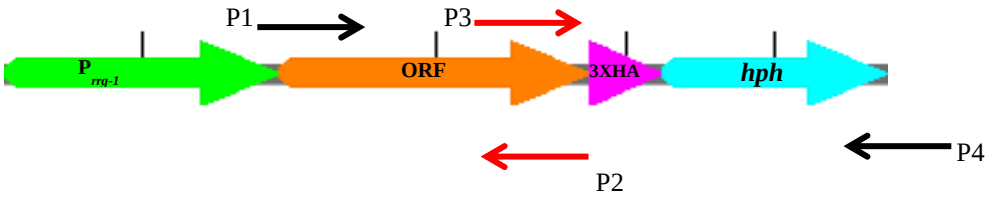
Expectation for RRG-1 phosphorylation site mutants

The possible results for strains containing mutations to block phosphorylation of RRG-1 are summarized in Table 2. While in yeast cells, the change of aspartate to glutamate prevented phosphorylation of RRG-1, it is possible that this change may mimic phosphorylation in *N. crassa*.

Table 2 Summary of expected results for levels and rhythms in P-OS-2 in the RRG-1 mutants

1. Rhythms in phosphorylation of OS-2	(1) If the phosphorelay is important for P-OS-2 rhythms: (a) In a constitutively phosphorylated RRG-1 strain, low levels of P-OS-2 will be observed. (b) In an un-phosphorylated mutant, amplitude of P-OS-2 rhythms will be higher than WT (2) If the phosphorelay is not important for P-OS-2 rhythms P-OS-2 rhythms will not change.
2. Levels of phosphorylation of OS-2	A constitutively active RRG-1 mutant will be lethal due to over-activation of OS-2; however if the mutant is constitutively inactive, the mutant will be viable. This result will depend on the phosphorylation state of the aspartate to alanine and aspartate to glutamate substitutions. Depending on the phenotype of the resulting strain, the phosphorylation status can be predicted.

A



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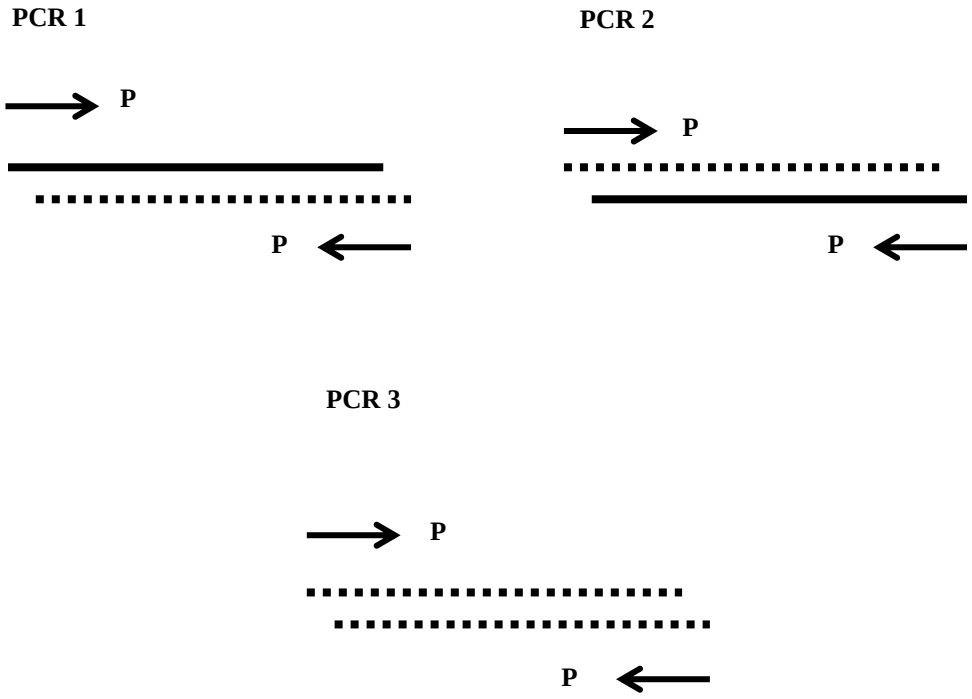


Figure 3 PCR-based site-directed mutagenesis method to construct phosphorylation site-specific mutations in RRG-1. (A) Schematic representation of the construct design containing the mutations. (B) Steps involved in the PCR-based site-directed mutagenesis method. Primers P1 and P4 are universal primers. Primers P2 and P3 are mutagenized primers. The template DNA for the first round of PCR used was the genomic DNA from a strain containing an HA tagged version of RRG-1. (C) Picture of the gel showing the amplification of the full length product after PCR 3. Lane 1 corresponds to the fragment containing D to E mutation and lane 2 corresponds to the fragment containing D to A mutation**.**

C

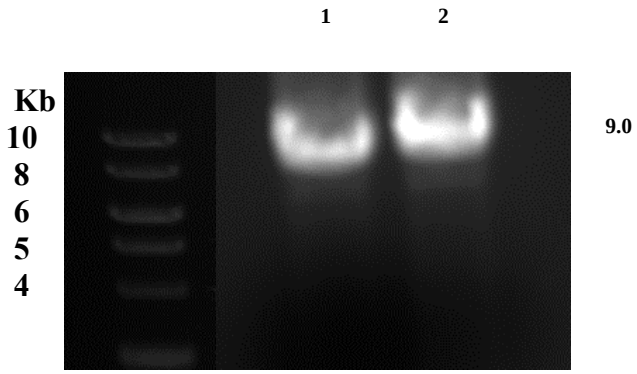


Figure 3 (Continued)

3.3 Construction of RRG-1::LUC reporter

Previous work demonstrated that HPT-1 mRNA and protein levels accumulate with a circadian rhythm (43). However, it was not known if *rrg-1* mRNA and RRG-1 protein accumulate with a circadian rhythm. To determine the rhythmicity of RRG-1, a reporter strain expressing a RRG-1::LUC translational fusion construct was generated. Luciferase activity was measured in RRG-1::LUC cells grown in DD at 25˚ C for 5 days. I found that RRG-1::LUC levels were rhythmic with a period of 21.93 h (Figure 4). Further validation of circadian rhythmicity of the strain needs to be done by assaying the levels in a clock mutant strain.

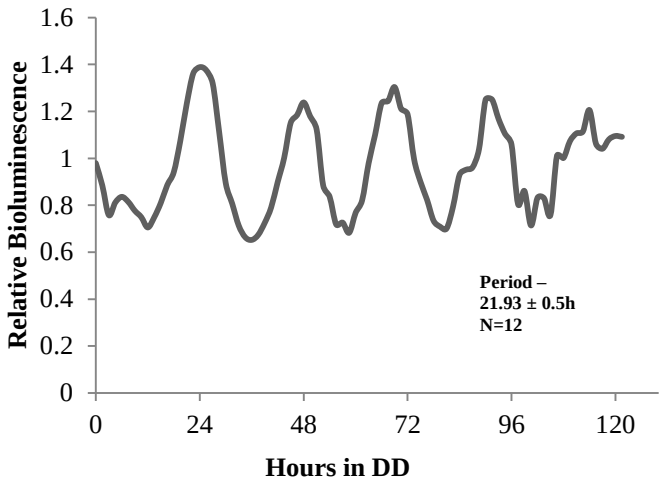


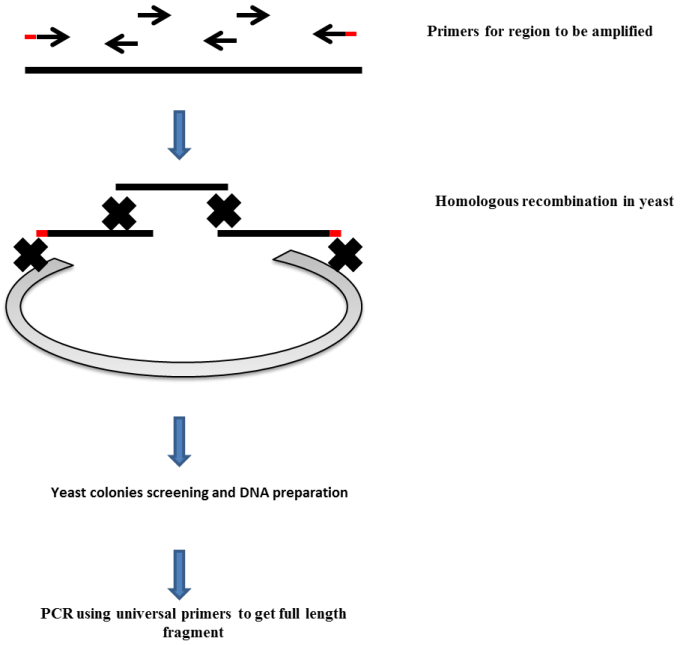
Figure 4 Rhythms in RRG-1::LUC. Luciferase trace showing rhythms in RRG-1::LUC levels. The average of 12 replicates is plotted and the data was normalized to mean and baseline de-trended using BioDare analysis (80).

3.4 Construction of $P_{rrg\text{-}1::luc}$ transcriptional reporter

To determine if *rrg-1* transcripts accumulate rhythmically, a reporter construct was generated in which the expression of the luciferase gene was controlled by the *rrg-1* promoter. This construct was designed for integration at the *csr* locus and selection for cyclosporine resistance. The cyclosporine resistance-based gene replacement strategy was previously described in *N. crassa* (81). A yeast-based recombinational cloning strategy was used to generate the reporter construct as described previously. This method

relies upon the endogenous homologous recombination machinery in yeast, which can ligate fragments with compatible ends. Using a PCR-based strategy; fragments with homologous ends can be generated for recombination in yeast, thus eliminating the need for additional manipulation, such as restriction digestion and ligation. An overview of the protocol to construct the promoter luciferase transcriptional fusion is shown in Figure 5A and the design of the construct is shown in Figure 5B. The outer most primers have homologous sequences to the yeast shuttle vector. In the second step, the amplicons are generated by PCR. In the third step, the amplicons, along with the restriction-digested shuttle vector, are transformed into yeast. In the final step, yeast colonies are screened for the ligated fragment and then the full fragment is amplified from the positive colonies. Figure 5C shows the full length product of expected size (7 kb) from the positive yeast colonies. Unfortunately, attempts to transform this fragment into *N. crassa* cells and select cyclosporine resistance were unsuccessful.

A



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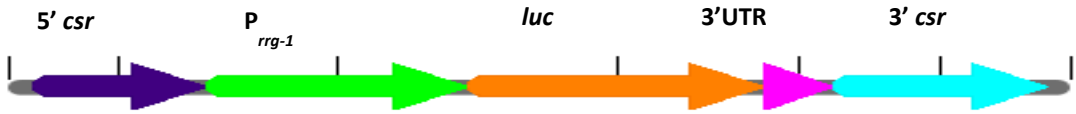


Figure 5 Construction of Prrg-1::luc. (A) Schematic representation of the yeast recombinational cloning protocol used to construct the reporter. (B) Design of the P_{rrg-1}::luc reporter construct targeted to the *csr* locus in *N. crassa*. (C) Picture of the agarose gel showing amplification of the full length fragment from yeast colonies corresponding to the total size of 7.0 Kb. Lanes 1 and 2 correspond to amplification of the fragment from two different yeast clones.

C

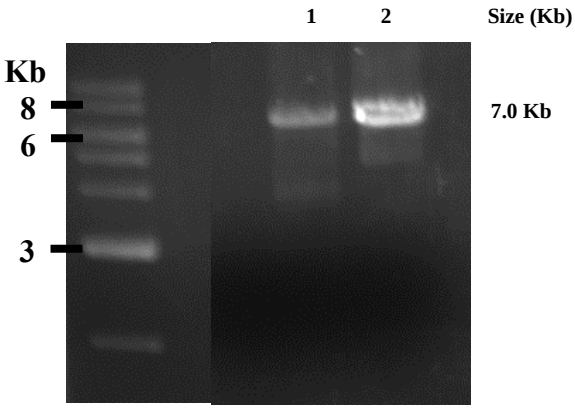


Figure 5 (Continued)

3.4 Phosphorylation of RRG-1

Phosphorelays function by transfer of phosphate groups from a sensor histidine kinase to a response regulator via a histidyl phosphotransferase protein. The phosphorylation status of the response regulator protein determines the state of signal transduction to the MAPK cascade. The purpose of this experiment was to detect the phosphorylation state of RRG-1 by using commercially available phos-tag acrylamide combined with SDS-PAGE and followed by Western blotting. I used an available strain in which RRG-1 is tagged with an HA epitope tag. λ-phosphatase treatment was carried out to confirm the phosphate-specific signal. Total proteins were separated on phos-tag SDS-PAGE gels

(Figure 6) and RRG-1 was detected using an anti HA antibody. Phos-tag gels were successfully used to detect the phosphorylated form of RRG-1::HA which is indicated by the shift in the band. This is because of the fact that phosphorylation leads to an increase in molecular weight of the protein. In the samples that were treated with the enzyme, the shifted band is not observed confirming that this band is specific to the phosphorylated RRG-1::HA. In the samples that were treated with phosphatase inhibitors, the phosphorylation specific signal is observed further confirming the specificity of the signal. In future studies, this protocol can be utilized to determine if phosphorylation of RRG-1 is clock-controlled.

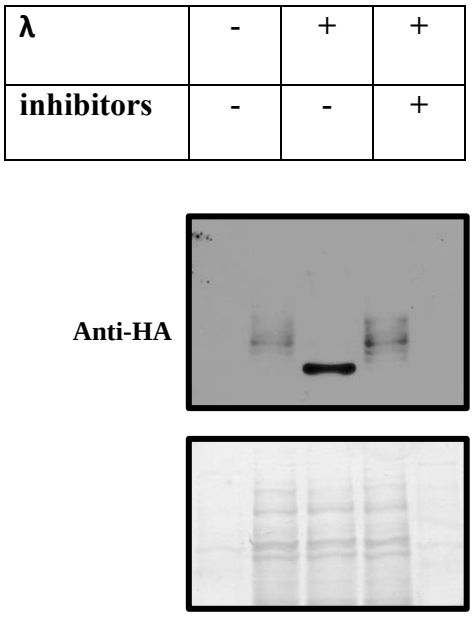


Figure 6 Phos-tag gels showing P-RRG-1. Western blots of proteins from a RRG-1::HA-tagged strain. 50 µg total proteins treated with (+) or without (-) λ-phosphatase or λ-phosphatase inhibitors were loaded. Amido black stain of the membrane was used as loading control.

3.5 Immunoprecipitation of HPT-1::FLAG

In plants and fungi, two component systems function by employing a phosphorelay mechanism whereby a phosphate group is transferred from the sensor histidine kinase protein via a histidyl phosphotransferase to a response regulator protein. The genome sequence of *N. crassa* revealed the presence of 11 hybrid histidine kinase proteins (82). How these HK proteins might signal to (HPT-1) and to the response regulator proteins (RRG-1 was not known. Furthermore, RRG-2, a second potential response may also participate in signaling downstream of HPT-1 (62). I hypothesized that the single HPT-1 protein forms a bottleneck in the phosphorelay mechanism, receiving signaling inputs from multiple upstream histidine kinases involved in environmental and stress signaling pathways. One test of this hypothesis is to determine if HPT-1 interacts with components of other signaling pathways. This could be accomplished by immunoprecipitating HPT-1 to determine what proteins it interact s with it by mass spectrometry. The purpose of this experiment was to immunoprecipitate HPT-1 protein in order to identify the potential binding partners of HPT-1. An available strain (DBP 1165) containing a tagged version of HPT-1 protein in which the *hpt-1* gene was replaced by a HPT-1::FLAG fusion construct was used (43). WT (FGSC 2489) was used as control (Figure 7).

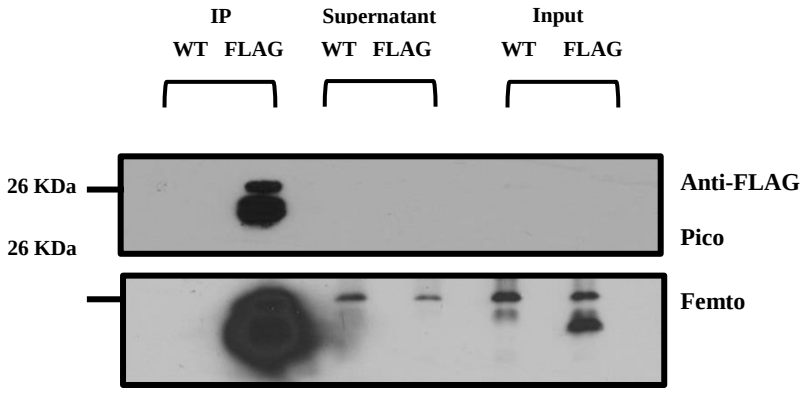


Figure 7 Immunoprecipitation of HPT-1::FLAG. Western blots probed with anti-FLAG antibody are shown. IP, supernatant and input samples from WT and HPT-1::FLAG tagged strains were used for analysis. Supernatant was collected after incubating the beads with the protein. IP represents the eluted proteins that were bound to the beads. The expected size of HPT-1::FLAG is approximately 25 KDa. Immunoreactivity was visualized with a Super Signal West Pico chemiluminescence substrate. Additionally, Super Signal West Femto substrate was used to show the band corresponding to HPT-1::FLAG in the input. In the supernatant, however, this band is not detected suggesting that most of the proteins were bound to the beads.

3.6 Sucrose gradient centrifugation to purify components of the phosphorelay

To determine if the components of the phosphorelay are involved in protein complexes, sucrose density gradient centrifugation was carried out. When subjected to density gradient centrifugation technique, the medium exhibits a concentration gradient increasing in the direction of the centrifugal force and hence the molecules separate in the layers based on their densities. Thus, higher molecular weight protein complexes settle at the bottom of the gradient, whereas individual proteins of lower molecular weight remain in the top layers. The protocol is schematically represented in Figure 8A. Figure 8B shows the results from the western blot analysis of HPT-1::FLAG, RRG-1::HA and RRG-2::V5 strains. The results indicate that both RRG-1::HA and RRG-2::V5 may be involved in protein complexes. It is also seen that these proteins appear in the same fractions. It is possible that these may be interacting with each other. This is in agreement with the predicted protein sequences of RRG-1 and RRG-2 proteins which both have an intermolecular recognition domain which is known to be involved in protein-protein interactions.

A

B

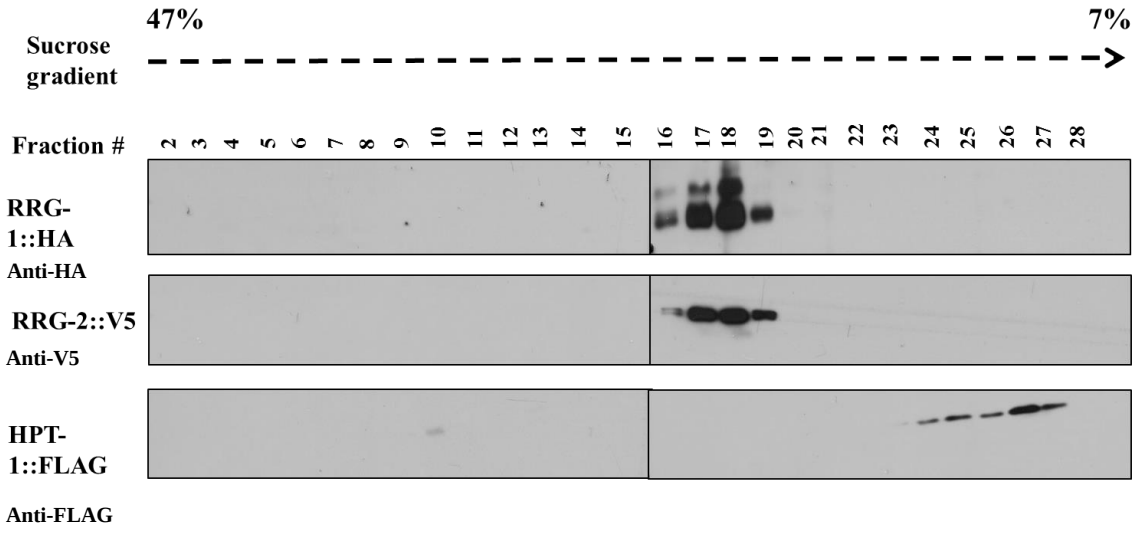


Figure 8 Sucrose gradient centrifugation of RRG-1::HA, RRG-2::V5 and HPT-1::FLAG. (A) Schematic representation of the sucrose density gradient centrifugation protocol. (B) Western blot analysis of fractions collected after ultracentrifugation using the indicated antibodies to detect RRG-1, RRG-2, and HPT-1.

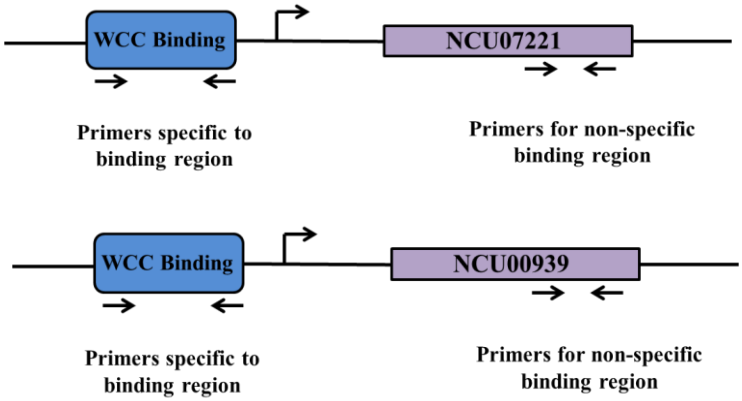
3.7 Role of clock-controlled histidine kinases in the circadian regulation of the phosphorelay

In the OS pathway phosphorelay, OS-1 is the sensor histidine kinase that transduces information on the state of the environment to the downstream phosphorelay. From previous work, it was shown that *os-1* mRNA and OS-1 protein do not accumulate rhythmically (43). The *N. crassa* genome is predicted to have 10 other histidine kinase proteins; however, only few of these are previously characterized (6, 82). Two of the predicted histidine kinase proteins (NCU07221 and NCU00939) were identified as direct targets of the WCC using ChIP-seq, suggesting the possibility that they may be clock-controlled (37). In a typical two component system, histidine kinases interact with the HPT-1 protein to mediate signal transduction. *N. crassa* contains only one HPT protein; therefore, I hypothesized that clock-controlled histidine kinases contribute to rhythmic activation of the phosphorelay.

To test this hypothesis, I first validated WCC binding to the promoters of *ncu07221* and *ncu00939* using an independent ChIP in *N. crassa* strains, *bd*⁻ (DBP 369) and *bd*⁻, *Δwc-2* (DBP 257). Following the ChIP, qPCR was used to determine if the WC-2 binds to the promoters of the two histidine kinase genes.). The design of primers for the binding sites and non-binding sites for NCU07221 and NCU00939 is shown in Figure 9 (A). The results for 3 technical replicates are shown in Figure 9 (B). I found enrichment in the amount of DNA corresponding to the primers specific for the WCC binding region in the *ncu07221* promoter. PCR with primers corresponding to the non-specific internal control

sequence shows no enrichment in DNA. In the *Δwc-2* strain, no enrichment is seen. This data independently validates the binding of WCC to the promoter of ncu07221. Similar to *ncu07221*, *ncu00939* is also a target of WCC as seen from Figure 9 (C). Further, my results indicate that the WCC binding at the promoters of *ncu07221* and *ncu00939* is enriched after 15 min and 30 min of light induction.

A



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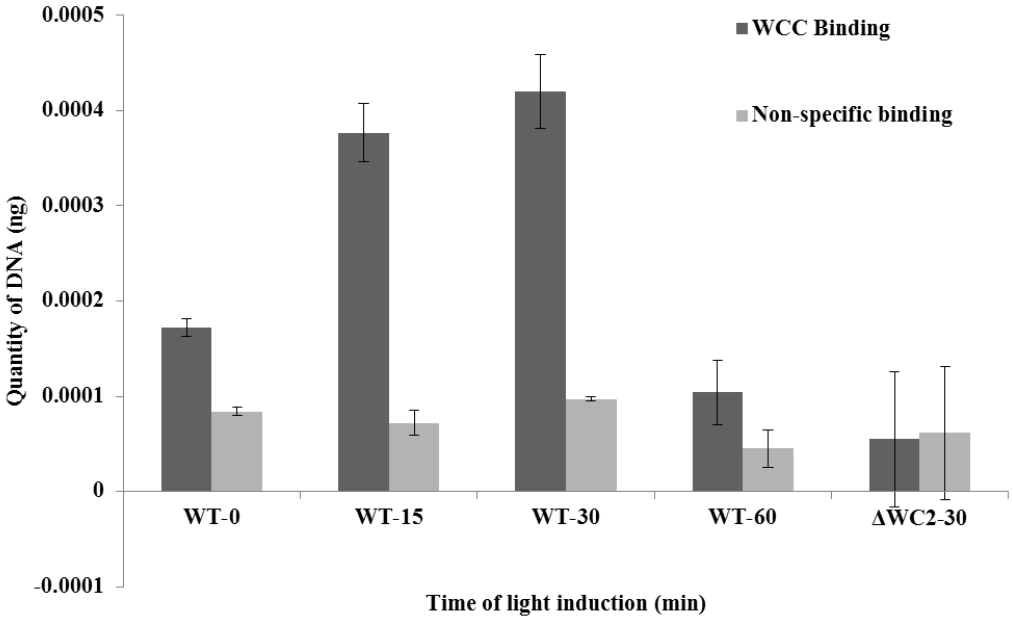

Figure 9 NCU07221 and NCU00939 are direct targets of WCC. (A) Strategy for primer design for qPCR. (B) qPCR data showing DNA enrichment (ng) for binding of WCC at the promoter of *ncu07221* and at a non-specific internal region in the gene. (C) qPCR data showing DNA enrichment (ng) for binding of WCC at the promoter of *ncu00939* and at a non-specific internal region in the gene.

C

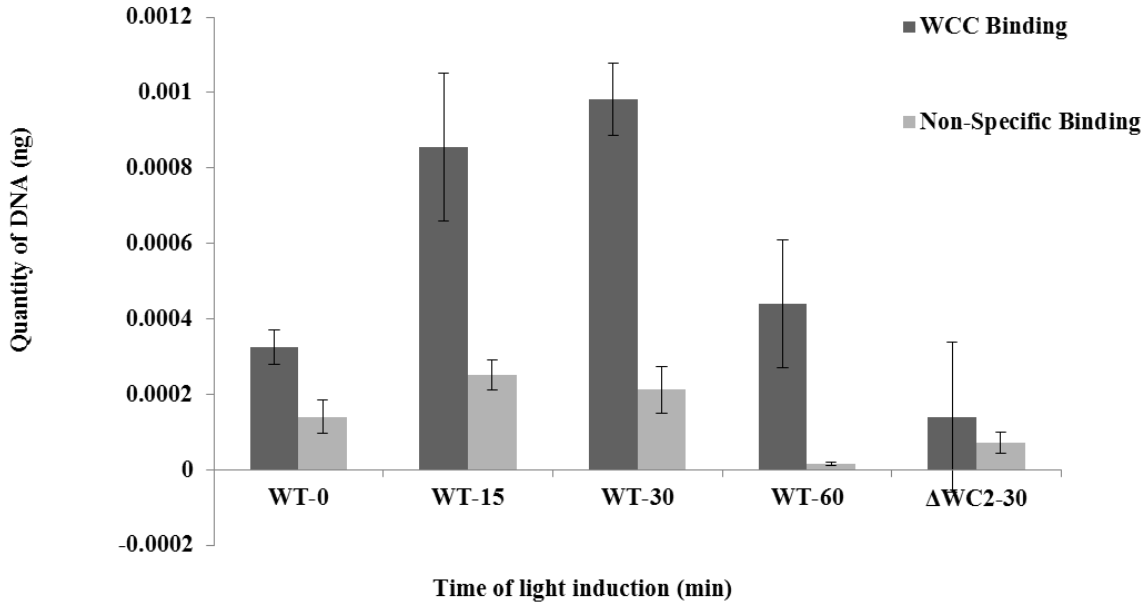

Figure 9 (Continued)

3.8 Tagging NCU07221 and NCU00939

Preliminary data indicated that the mRNA levels of *ncu07221* and *ncu00939* accumulate with a circadian rhythmic (Goldsmith CS, unpublished data). To validate clock regulation of NCU07221 and NCU00939, I generated constructs to epitope tag NCU07221 and NCU00939 with HA using 3-way PCR. The constructs were transformed into wild type *N. crassa* cells (FGSC # 2489) to replace the native genes with the tagged versions by homologous recombination. The strains were stored and are now available to determine if NCU07221::HA and NCU00939::HA accumulate with a circadian rhythm. The tagged proteins can also be used in the future to detect interactions with other components of the OS pathway phosphorelay by immunoprecipitation.

4. SUMMARY

Circadian regulation of the activity of MAP kinase pathways provides a mechanism for organisms to prime the stress response to the time of day when the cells are more prone to stressful conditions. It was previously demonstrated that the circadian clock regulates the activity of the MAPK OS-2 in *N. crassa*, and that this regulation occurs by direct transcriptional control of the MAPKKK *os-4* by the WCC (43). However, it was also found that the clock impinges upon the OS pathway at multiple levels. HPT-1, a central component of the phosphorelay, was shown to be rhythmic both at the level of mRNA and protein abundance (43). In addition, this work demonstrated that RRG-1 protein levels accumulate rhythmically. A transcriptional reporter construct was also generated in which the luciferase gene was put under the control of the *rrg-1* promoter. In the future, this construct will be transformed in WT and clock mutant *N. crassa* cells to determine if *rrg-1* mRNA is also clock-regulated or if rhythmic RRG-1 protein accumulation is due to posttranscriptional regulation.

Studies to understand the phosphorelay mechanisms have been challenging due to the lack of commercially available antibodies for detection of the histidine and aspartate phosphorylation. This is likely due to the transient nature of the phosphorylation events. However, tools such as phos-tag acrylamide coupled with SDS-PAGE have been successfully used to detect the phosphorylation of response regulator proteins in bacterial systems (83). In this study, I was able to detect phosphorylation of RRG-1::HA protein using the phos-tag SDS-PAGE technique. This protocol can be used in the future

to determine if phosphorylation of RRG-1::HA is rhythmic under constant conditions in WT and clock mutant cells. In addition, a role for upstream components of the phosphorelay in rhythmic accumulation of phosphorylated RRG-1 can be determined using the appropriate mutant strains.

The rhythmic regulation of *os-4* mRNA by the circadian clock is necessary for rhythms in activity of P-OS-2; however, the role of clock regulation of the phosphorelay in OS-2 activity rhythms is not understood. The phosphorelay is required for acute activation of the MAPK in the presence of osmotic stress (50, 63). However, it is not clear if the phosphorelay is required for rhythmic activation of OS-2 under constant environmental conditions. To test this possibility, I constructed phosphorylation site-specific mutant fragments of RRG-1::HA in which the conserved aspartate residue (D-921) is either substituted by alanine or glutamate. The alanine substitution was expected to render the protein non-phosphorylatable and the glutamate substitution was expected to act as a constitutively phosphorylated version. Attempts to transform these fragments into *N. crassa* WT strain did not yield viable transformants. This could be due to the fact that the OS-2 MAPK was hyper-activated in both types of substitution mutants, suggesting that these mutations render the protein non-phosphorylatable as is the case with yeast Hog-1p (76). Further validation of this idea needs to be done by transforming the mutant fragments into a non-functional version of OS-2 and assaying the levels of upstream components such as OS-4 and OS-5.

The *N. crassa* genome contains 11 hybrid histidine kinases, 1 HPT protein and 2 response regulator proteins. Most of the histidine kinases are uncharacterized (82). Since histidine kinases are known to interact with HPT-1, I hypothesized that HPT-1 could be functioning as a central component in a bottleneck of signal transduction pathways receiving signals from a number of upstream histidine kinases and relaying these signals to the appropriate effector molecules. Clock control of HPT-1 levels could therefore provide a mechanism for circadian regulation of pathways other than the OS pathway. To test this possibility, I proposed the idea of IP HPT-1 and carrying out mass spectrometry to identify potential binding partners of HPT-1. In this study, IP of HPT-1::FLAG was successfully performed at LL 30˚ C using an affinity gel. Using this protocol, IP of HPT-1::FLAG can be carried out at different conditions (DD and stress) to determine the binding partners of HPT-1. This will provide insights into what other pathways are regulated by HPT-1.

As previously mentioned, the role of histidine kinases in stress and circadian responses is mostly uncharacterized in *N. crassa*. From the ChIP-seq of WCC, two putative histidine kinases were suspected to be targets of the WCC (37). Since histidine kinases are known to interact with HPT proteins, we want to test the possible role of the clock-controlled histidine kinases in the circadian regulation of the phosphorelay. An independent ChIP was performed to validate the binding of WCC at the promoters of *ncu07221* and *ncu00939*. To further understand their role in circadian regulation, the proteins were tagged with an HA epitope tag and the tagged versions can be transformed

into WT and clock mutant cells used to determine if the proteins accumulate with a circadian rhythm.

To determine if NCU07221 and NCU00939 are involved in the circadian regulation of the phosphorelay, knockouts of these histidine kinases were crossed to a strain containing tagged version of HPT-1::FLAG. The purpose was to detect if HPT-1 phosphorylation rhythms are affected in the knockouts of the clock-controlled histidine kinases. Since the phosphorylation of HPT-1 plays a major role in signal transduction in the phosphorelay mechanism, I predicted that if the hisitidine kinases NCU07221 and NCU00939 are involved in circadian regulation of the phosphorelay, HPT-1 phosphorylation (both levels and rhythms) will be affected in the *ncu07221* and *ncu00939* knockouts. Currently no antibodies are available to detect the phosphorylation of HPT-1. Phosphorylation of HPT-1 was also not successfully detected by phos-tag SDS-PAGE gels. Therefore, the role of NCU07221 and NCU00939 in the circadian regulation of the phosphorelay is still unknown. In the future, if there are antibodies available to detect phosphorylation of the histidine kinases and HPT-1 proteins, the above strains can be used to determine the effect of circadian regulation of NCU07221 and NCU00939 on the OS pathway phosphorelay.

Similar to the OS pathway in *N. crassa*, the clock controls the activity of the p38 pathway in mammals (84) (Goldsmith, Bell-Pedersen *et. al*., unpublished data). The p38 MAPK pathway in mammals has been implicated in numerous stress responses

including UV damage, inflammation and heat. Thus, understanding the mechanistic operations of the OS pathway in *N. crassa* might provide insights into the details of the mechanism of stress response by the p38 MAPK pathways in higher eukaryotes. Understanding how the phosphorelay activates the MAPK module would additionally provide useful information about activation of MAPK cascades in mammals. Lastly since the phosphorelay mechanism is not present in animals, components of the phosphorelay make attractive antifungal drug targets.

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